Migeon, C. D. & Plager, J. E. (1955). *J. clin. Endocrin.* **15**, 702.
Munson, P. L. & Kenny, A. D. (1954). *Recent Progr. Hormone Res.* **9**, 135.
Neunhoeffer, O., Thewalt, K. & Zimmermann, W. (1961). *Hoppe-Seyl. Z.* **323**, 116.
Norymberski, J. K. (1952). *Nature, Lond.*, **170**, 1074.
Werbin, H. & Ong, S. (1954). *Analyt. Chem.* **26**, 762.
Zimmermann, W. (1935). *Hoppe-Seyl. Z.* **233**, 257.
Zimmermann, W. (1936). Ph.D. Thesis: Bonn.
Zimmermann, W. (1937). *Hoppe-Seyl. Z.* **245**, 47.
Zimmermann, W. (1955). *Hoppe-Seyl. Z.* **300**, 141.
Zimmermann, W., Anton, H.-V. & Pontius, D. (1952). *Hoppe-Seyl. Z.* **289**, 91.

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Incorporation of Amino Acids into the Protein of Isolated Mitochondria

A SEARCH FOR OPTIMUM CONDITIONS AND A RELATIONSHIP TO OXIDATIVE PHOSPHORYLATION

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Most of the protein biosynthesis occurring in rat-liver cells takes place in the microsomes (Keller, Zamecnik & Loftfield, 1954), but some amino acid incorporation into protein also occurs in nuclei (Rees & Rowland, 1961) and in mitochondria (McLean, Cohn, Brandt & Simpson, 1958; Roodyn, Reis & Work, 1961). The amount of amino acid incorporation into protein which occurs in isolated mitochondria is very small compared with that found with microsomes (McLean *et al.* 1958), but nevertheless the mitochondrial system is of special interest because of the importance of the mitochondria in the general metabolism of the cell.

Since only a slight amount of incorporation occurs in mitochondria it seemed desirable to confirm the earlier reports on mitochondrial amino acid incorporation (McLean *et al.* 1958) and to establish that the mitochondria themselves are responsible for the incorporation of amino acids into protein. The optimum conditions for the incorporation of amino acids into the proteins of isolated rat-liver mitochondria and the effect of variation of the conditions on the rate of incorporation were also investigated.

METHODS

Animals. Female albino rats, weighing about 200 g., were used in the experiments.

Radioactive compounds. DL-[1-^{14}C]Leucine and generally (G) labelled L-[G-^{14}C]leucine were used, and were obtained from The Radiochemical Centre, Amersham, Bucks.

Materials. Free acid AMP, the sodium salts of ATP, ADP, and AMP, tris and NAD were obtained from the Sigma Chemical Co. Ribonuclease (recrystallized five times) was obtained from both the Sigma Chemical Co. and Nutritional Biochemicals Corp. Phosphocreatine was prepared by the method of Ennor & Stocken (1948), and phosphocreatine kinase by the method of Kuby, Noda & Lardy (1954). Bovine serum albumin prepared by Armour Pharmaceuticals was used and rat serum albumin was prepared by the method of Korner & Debro (1956). All other reagents were of AnalaR grade, except amino acids, which were obtained from Roche Products Ltd. Sucrose was further purified by passing solutions through a column of Amberlite MB-1 mixed-bed ion-exchange resin, and the solutions were then boiled to remove dissolved carbon dioxide and to reduce bacterial contamination. All solutions were made in glass-distilled water.

Preparation of mitochondria. The rats were killed by decapitation and were bled. The liver was rapidly removed and transferred to ice-cold 0·25M-sucrose, in which it was cut into small pieces with scissors. All subsequent operations in the preparation of the mitochondria were carried out between 0° and 2°. The pieces of liver were blotted, the volumes measured by displacement in fresh 0·25M-sucrose and the liver was homogenized in 0·25M-sucrose, a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept in an ice bath, being used.

Homogenization was carried out in three stages as described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The liver was first homogenized in 2·5 vol. of sucrose, with three strokes of the homogenizer, and was then filtered through Terylene net (1 mm. × 1 mm. mesh) to remove fibrous connective tissue. The homogenate was then centrifuged at 600*g* for 10 min. to remove nuclei and intact cells. The supernatant fluid was kept at 0° and the pellet was rehomogenized in 1·5 vol. of 0·25M-sucrose and spun again at 600*g* for 10 min. This step was repeated once more with 1 vol. of 0·25M-sucrose, so that the liver was homogenized three times in all, in a total volume of 0·25M-sucrose equal to five times the volume of liver used, and on all occasions the supernatant fluid was removed and kept. The combined supernatants were then centrifuged at 5000*g* for 10 min. to give a mitochondrial pellet. The super-

natant fluid was removed and discarded. The method of homogenization used gives a high yield of mitochondria, and is intended to prevent damage to the mitochondria.

Purification of the mitochondria. Method 1: The mitochondrial pellet was resuspended in 0·25M-sucrose by gentle use of the homogenizer, and was centrifuged at 600*g* for 10 min. The supernatant was removed and centrifuged at 3250*g* for 10 min., and the pellet was resuspended in 0·25M-sucrose. This step was repeated five times, so that in all the mitochondria were given six washes. At the end of this procedure the supernatant was quite clear, indicating that the cell sap and microsomes had been removed.

Method 2: The mitochondrial pellet was resuspended in 0·25M-sucrose and centrifuged at 600*g* for 10 min. The supernatant fluid was removed and portions (10 ml.) were carefully layered on 10 ml. of 0·5M-sucrose in 50 ml. centrifuge tubes. These tubes were then centrifuged at 3250*g* for 10 min. in a swinging-bucket centrifuge, and the upper layer was pipetted off. The pellet, now in dense sucrose, was diluted with about 40 ml. of 0·25M-sucrose and resuspended by use of the homogenizer. It was then centrifuged at 5000*g* for 10 min. and the pellet was resuspended in 10 ml. of 0·25M-sucrose. The washing by layering was repeated.

The mitochondria obtained by either of these methods were suspended in solutions of either sucrose or sucrose with tris and salts so that when they were finally added to the incubation medium the concentrations of solutes were those described below.

Preparation of microsomes. Rat liver was homogenized with 5 vol. of 0·25M-sucrose in the way described above and the homogenate was centrifuged at 10 000*g* for 10 min. to remove mitochondria and nuclei. The supernatant fluid was then centrifuged at 105 000*g* for 1 hr. and the pellet of microsomes was resuspended by the use of an homogenizer in medium A (0·15M-sucrose, 35 mM-tris, pH 7·4, 25 mM-KCl).

Preparation of cell sap. Rat liver was homogenized with 2 vol. of 0·25M-sucrose in the way described above and the homogenate was centrifuged at 5000*g* for 10 min. The supernatant fluid was removed and centrifuged at 105 000*g* for 1½ to 2 hr., and the final supernatant of cell sap was then carefully pipetted off, leaving both the microsomal pellet and also the layer of lipid which forms at the top of the centrifuge tube.

Incubation medium. The composition of the incubation medium varied from one experiment to another as the effect of different conditions was examined but some components of the medium remained constant (medium A: 0·15M-sucrose, 35 mM-tris, pH 7·4, 25 mM-KCl). The further additions are described in the Tables and Figures. Sometimes a more complex medium was used (medium B: medium A plus 10 mM-nicotinamide, 0·25 mM-NAD and 1 mg. of synthetic amino acid mixture/ml.). The pH of any additions to the incubation medium was adjusted to pH 7·4 where necessary by the addition of NaOH, except in those experiments in which the concentration of Na^+ ions was varied, when the pH of the medium was adjusted by the addition of tris. The pH of the final incubation mixture was checked and found to be 7·4. The synthetic amino acid mixture had the following molar proportions: alanine 0·6, arginine 0·2, aspartic acid 0·4, asparagine 0·2, cysteine 0·2, glutamic acid 0·7, glutamine 0·25, glycine 1·0, histidine 0·1, lysine 0·5, methionine 0·3, phenylalanine 0·25, proline 0·35, threonine 0·4, tyrosine 0·3, valine 0·35. The L-isomer of the amino acids was used in each case, except that of glycine.

Conditions of incubation. Unless otherwise stated, incubation was at 37°, in air, and with shaking. Each 1 ml. of incubation medium contained the mitochondria obtained from 1 g. of liver. This is equivalent to approx. 6 mg. of mitochondrial protein.

Preparation of protein samples. The reaction was stopped by the addition of 0·5N-$HClO_4$ containing unlabelled amino acid corresponding to the radioactive amino acid used. The precipitated protein was then washed with 0·5N-$HClO_4$ containing the unlabelled amino acid and dissolved in N-NaOH containing unlabelled amino acid and allowed to digest at room temperature for at least 1 hr. to remove RNA and amino acids bound to, but not incorporated into, the protein. After acidification with 6N-HCl, the protein was centrifuged down and re-extracted with N-NaOH. The protein was reprecipitated by acidification and was boiled for 5 min. with 0·5N-$HClO_4$. The protein was treated twice with chloroform–ethanol–ether (1:2:2, by vol.) to remove the lipids and the powder was dried by two washes of ether–light petroleum (b.p. 60–80°) (3:1, v/v). The dried powder was dissolved in 0·3 ml. of 95% formic acid.

Stainless-steel planchets with concentric ridges were weighed and the protein solution in formic acid was poured on to the centre of the planchet. It spread over the planchet, filling each of the concentric grooves in turn, and each protein sample occupied a constant area of 4·55 $cm.^2$. The formic acid of the samples was evaporated under a heat lamp and the planchets were then re-weighed.

Measurement of radioactivity. Radioactivity of the samples was estimated with a gas-flow proportional counter with an end window ($< 0·15$ mg./$cm.^2$). Sufficient counts were recorded to ensure a standard error of less than 3% and corrections were made for background counts and for self-absorption.

Correction for cell-sap protein. When cell sap had been added to the incorporation system, specific activities were corrected to give counts/min./mg. of mitochondrial protein. This was done by determining the protein content of both the cell sap and of the mitochondria as described below and multiplying the specific activity obtained by mg. of total protein/mg. of mitochondrial protein. The figures obtained by use of this correction were similar to the specific activities obtained in those experiments when the mitochondria were separated again at the end of the incubation and prepared and counted separately from the soluble part of the incubation mixture.

Determination of ribonucleic acid. RNA was separated from the other components of the samples by treatment with N-NaOH (Schneider, 1946) to give RNA-hydrolysis products and a precipitate of protein. The RNA was then estimated from the extinction at 260 and 286 mμ according to the method of Tsanev & Markov (1960).

Determination of protein. The protein content of samples was determined from the nitrogen content of the final pellet after acid-soluble material and RNA had been removed by the method of Schneider (1946). The protein pellet was dissolved in nitrogen-free H_2SO_4 and estimated by the micro-Kjeldahl technique, with purified mitochondrial protein as a standard. RNA content was expressed in terms of the protein content of the sample and

both estimations were made on the same sample. This reduces errors due to manipulative losses during RNA separation which occur if the RNA estimation is made on a separate sample.

Determination of free amino acids of mitochondria. Mitochondria prepared and washed in the usual way were treated with trichloroacetic acid to a final concentration of 5% and the protein precipitate was centrifuged down. The supernatant was removed and extracted eight times with ether to remove trichloroacetic acid. Total amino nitrogen in this material was estimated by the ninhydrin method of Chibnall, Mangan & Rees (1958). The amino acids were further purified by absorption on to Amberlite IR-120 resin (H^+ form) and subsequent elution with aq. 5N-NH_3 soln. The amino acid mixture was then analysed on the Beckman Spinco automatic amino acid analyser.

Determination of P:O *ratios.* Oxygen uptake by the mitochondria was measured by means of the O_2 electrode (Chappell, 1961) and P:O ratios were determined by measuring the increase in uptake of O_2 caused by the addition of a known quantity of ADP, as described by Chance & Williams (1955). The O_2-electrode studies were carried out in a medium containing 20 mM-tris, pH 7·5, 60 mM-KCl, 12 mM-$MgCl_2$, 12 mM-KH_2PO_4 and 10 mM-sodium succinate.

RESULTS

Course of incorporation in time. When mitochondria are incubated at 37° in the presence of medium A plus 10 mM-$MgCl_2$, 10 mM-sodium succinate, 5 mM-AMP, 10 mM-KH_2PO_4, 0·5 μC of DL-[1-^{14}C]leucine and 0·2 ml. of cell sap/ml. of incubation mixture, they incorporate the radioactive amino acid into their protein. The progress of this incorporation is linear for about the first 40 min., and then there is a progressive decrease in the rate of incorporation. In some experiments the specific activity of the proteins decreased after about 2 hr.

Requirement for cell sap. In the initial stages of these investigations it was found that the addition of cell sap to the incubation mixture stimulated the amino acid incorporation into mitochondrial protein. The addition of 1 mg. of a mixture of amino acids (for composition see Methods section)/ml. reduced the dependence on cell sap, and when the concentration of inorganic ions was adjusted to the optima determined by the experiments described below and NAD and nicotinamide were added, amino acid incorporation into protein was found to be independent of the addition of cell sap.

Effect of inorganic ions. The studies on the effects of different concentrations of inorganic ions reported below were carried out in the absence of cell sap, since the salt content of the cell sap itself might make a significant contribution to the total ion content of the system.

(*a*) Mg^{2+} ions. The effect of Mg^{2+} ion concentration on the incorporation of amino acids into the protein of mitochondria incubated in medium B plus 10 mM-KH_2PO_4, 10 mM-potassium succinate, 5 mM-ATP or -AMP, and 0·5 μC of DL-[1-^{14}C]leucine/ml. was found to depend on which adenine nucleotide was present. If ATP was present the presence of as little as 0·5 mM-$MgCl_2$ reduced incorporation to 66% of that in the absence of added magnesium, and the inhibition continued almost linearly with increasing $MgCl_2$ concentration to 5·0 mM (see Fig. 2). When, however, AMP was added in place of ATP the addition of Mg^{2+} ions stimulated amino acid incorporation, with an optimum Mg^{2+} ion concentration of about 10 mM, as is shown in Fig. 2.

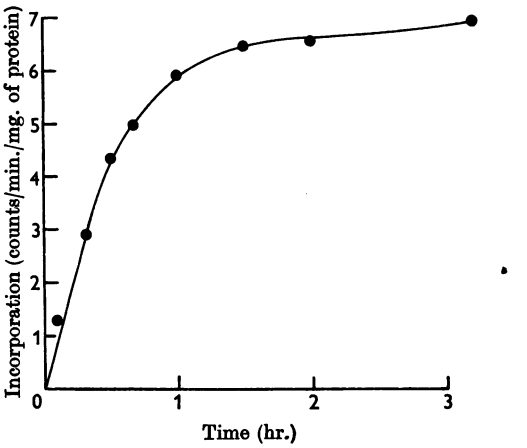

Fig. 1. Rate of incorporation of radioactive amino acids into mitochondrial protein. Mitochondria were incubated in medium A plus 10 mM-$MgCl_2$, 10 mM-sodium succinate, 5 mM-AMP, 10 mM-KH_2PO_4 and 0·5 μC of DL-[1-^{14}C]-leucine/ml. with 0·2 ml. of cell sap/ml. of incubation medium.

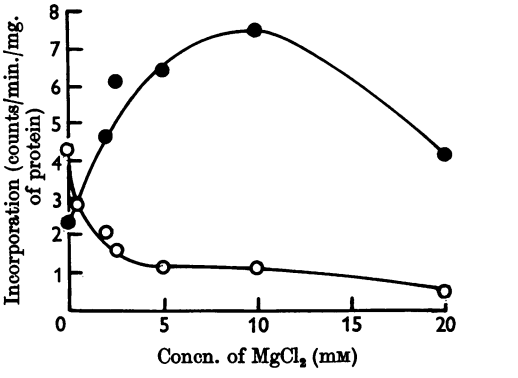

Fig. 2. Effect of different concentrations of $MgCl_2$ on amino acid incorporation. ○, In the presence of ATP; ●, in the presence of AMP. Mitochondria were incubated for 1 hr. in medium B plus 10 mM-KH_2PO_4, 10 mM-potassium succinate, 5 mM-ATP or -AMP and 0·5 μC of DL-[1-^{14}C]-leucine/ml.

The inhibitory effect of Mg^{2+} ions in the presence of ATP was studied at various incubation times. The effect of the Mg^{2+} ions on the course of incorporation of amino acids into protein is shown in Fig. 3. The rate of incorporation is reduced, and there is also a slight reduction in the duration of incorporation.

(*b*) Ca^{2+} ions. These ions are unable to replace Mg^{2+} ions in stimulating amino acid incorporation into mitochondrial protein, and are strongly inhibitory: 10 mM-$CaCl_2$ inhibits amino acid incorporation by 70–90 %, as is shown in Table 1.

(*c*) K^+ and Na^+ ions. The relative concentrations of K^+ and Na^+ ions has a considerable effect on the incorporation of amino acids into proteins by mitochondria. This is shown by Fig. 4, which presents the results of an experiment in which amino acid incorporation into the mitochondrial proteins was studied in a medium containing 0·15 M-sucrose, 35 mM-tris, pH 7·4, 10 mM-$MgCl_2$, 10 mM-nicotinamide, 0·25 mM-NAD, 1 mg. of synthetic amino acid mixture/ml., 10 mM-succinate, 10 mM-PO_4^{3-} ions, 50 mM-Cl^- ions, 5 mM-AMP (free acid) and 0·5 μc of DL-[1-^{14}C]leucine/ml., and in which the total molarity of K^+ and Na^+ ions was maintained at 70 mM and the concentration of the K^+ ions was varied from 0 to 100 % of the total of the K^+ and Na^+ ion concentrations. The amino acid incorporation rises with increasing K^+ ion concentration until the K^+ ions make up about 95 % of the total of K^+ and Na^+ ions. There is a fall in amino acid incorporation without added Na^+ ions. This experiment indicates that sodium is required for some process in the incorporation, but that maximal incorporation occurs when the potassium concentration predominates over that of sodium by about 9 to 1.

Effect of nucleotides. It was found that amino acid incorporation by the mitochondria at optimum rates required the addition of adenine nucleotides.

Table 1. *Effect of pretreatment with ribonuclease and of changes in the incubation medium on incorporation of amino acids into mitochondrial protein*

Mitochondria were incubated for 1 hr. in medium B plus 10 mM-$MgCl_2$, 5 mM-AMP, 10 mM-KH_2PO_4, 10 mM-sodium succinate and 0·5 μc of DL-[1-^{14}C]leucine/ml. Incorporation is expressed as a percentage of that found for the complete system.

	Incorporation (%)
Complete system	100
Preincubated at 37° for 30 min.	70
Preincubated with 0·5 mg. of ribonuclease/ml. at 25° for 30 min.	255
Incubated at 25° instead of 37° for 1 hr.	77
Amino acids omitted	79
AMP omitted	46
Magnesium replaced by 10 mM-$CaCl_2$	10
Reaction stopped at zero time	2

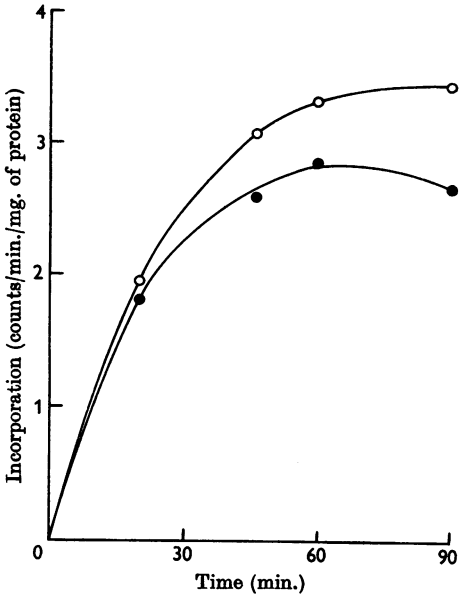

Fig. 3. Effect of magnesium on the course of incorporation in the presence of ATP: ○, without added magnesium; ●, plus 5 mM-$MgCl_2$. The mitochondria were incubated in medium A plus 20 mM-sodium succinate, 5 mM-ATP, 0·5 μc of L-[G-^{14}C]leucine.

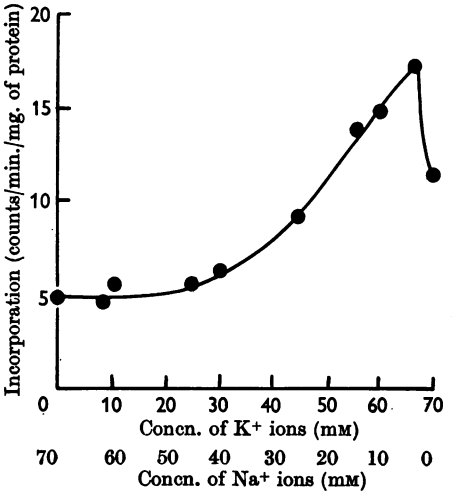

Fig. 4. Effect of different relative concentrations of Na^+ and K^+ ions on amino acid incorporation. Mitochondria were incubated for 1 hr. in 0·15 M-sucrose, 35 mM-tris, pH 7·4, 10 mM-$MgCl_2$, 10 mM-nicotinamide, 0·25 mM-NAD, 1 mg. of synthetic amino acid mixture (for composition see Methods section)/ml., 10 mM-succinate, 10 mM-PO_4^{3-} ions, 50 mM-Cl^- ions, 5 mM-AMP (free acid) and 0·5 μc of DL-[1-^{14}C]leucine/ml.

ATP, ADP and AMP were all effective, and the nucleotide that was most effective in stimulating amino acid incorporation depended on the other conditions of the system. In particular, the response depended on the Mg^{2+} ion concentration present, and each nucleotide worked best at a particular Mg^{2+} ion concentration, which differed from that required by the other nucleotides. At the optimum Mg^{2+} ion concentration AMP was more effective in stimulating amino acid incorporation into protein than were the other adenine nucleotides, even at their optimum Mg^{2+} ion concentration (Fig. 2).

Requirement for an energy source. In most of the experiments described here the mitochondria were incubated under conditions in which they were able to obtain energy by oxidative phosphorylation, and in these circumstances they were able to incorporate amino acids into proteins for more than 1 hr. (Fig. 5). Glutamate, as well as succinate, can serve as a substrate for the production of energy and will support the incorporation of amino acids into protein, but the glutamate-oxidizing system is more susceptible to damage than is the succinate-oxidizing system. Thus the absence of Mg^{2+} ions or vigorous homogenization of the mitochondria has a more drastic effect on incorporation when glutamate is used as the substrate than when succinate is the substrate (Table 2). Because of this greater sensitivity of the glutamate-oxidizing system, succinate was generally used as the substrate during these investigations.

The addition of serum albumin has been shown to stimulate oxidative phosphorylation in isolated mitochondria (Sacktor, 1953; Stern & Timonen, 1955). Table 3 reports the results of an experiment in which bovine serum albumin was added to the mitochondrial amino acid-incorporation system, and the effect on P:O ratios and the simultaneous ability of the mitochondria to incorporate amino acid into protein were examined. Bovine serum albumin was found to stimulate the amino acid incorporation when added either to fresh mitochondria or to mitochondria that had been pre-

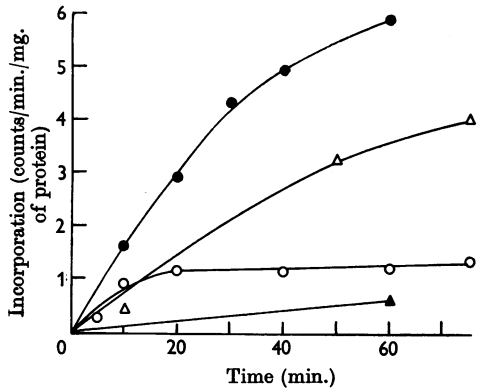

Fig. 5. Effect of different energy sources on incorporation of amino acids into microsomes and mitochondria. ○, Mitochondria; △, microsomes, incubated in medium A with 25 mM-creatine phosphate, 0·04 mg. of creatine phosphokinase/ml. and 5 mM-ATP; ●, mitochondria; ▲, microsomes, incubated in medium A with 10 mM-sodium succinate, 5 mM-AMP and 10 mM-KH_2PO_4. All incubation mixtures also contained 10 mM-$MgCl_2$, 0·2 ml. of cell sap/ml. of incubation mixture and 0·5 μC of DL-[1-^{14}C]leucine/ml.

Table 2. *Effect of different substrates on amino acid incorporation*

Mitochondria were incubated for 90 min. in medium A plus 5 mM-$MgCl_2$, 10 mM-AMP, 10 mM-KH_2PO_4, 10 mM-sodium glutamate or sodium succinate, 0·2 ml. of cell sap and 0·5 μC of L-[G-^{14}C]leucine/ml. of incubation mixture. Incorporation is expressed as a percentage of that found for the complete system with succinate as substrate.

	Incorporation (%)	
Substrate	Succinate	Glutamate
Complete system with normal mitochondria	100	122
Normal mitochondria, magnesium omitted	89	54
Complete system with vigorously homogenized mitochondria	101	109

Table 3. *Effect of serum albumin on amino acid incorporation into protein and on oxidative phosphorylation*

Mitochondria were incubated for 1 hr. in medium A plus 10 mM-$MgCl_2$, 5 mM-AMP, 10 mM-KH_2PO_4, 10 mM-sodium succinate, 0·2 ml. of cell sap and 0·5 μC of DL-[1-^{14}C]leucine/ml. of incubation mixture. Incorporation is expressed as a percentage of that obtained with fresh mitochondria with no further additions.

	Incorporation (%)			P:O ratio (obtained with fresh mitochondria with succinate as substrate)
	Fresh mitochondria	Mitochondria preincubated at 37° for 30 min.	Mitochondria stored at 0° for 24 hr.	
No further additions	100	98	118	2·06
With 1 mg. of bovine serum albumin/ml.	179	147	106	2·72
With 1 mg. of rat serum albumin/ml.	177	104	124	2·32

incubated at 37° for 30 min. There was also an increase in P:O ratio when bovine serum albumin was added to fresh mitochondria. Laudahn (1960) has suggested that homologous serum albumin may be more effective in restoring the P:O ratios of mitochondria than the heterologous albumin. The effect of rat serum albumin was therefore examined and the results are also shown in Table 3. No substantial differences were found between the effect of rat or bovine serum albumin on P:O ratios or on the incorporation of amino acids into mitochondrial protein.

Mitochondria were also incubated in the absence of an oxidizable substrate, but in the presence of creatine phosphate and creatine phosphokinase together with 5 mM-ATP. Incorporation of radioactive amino acids was then very slight and continued for only 15 min. (Fig. 5). When microsomes were incubated with creatine phosphate, creatine phosphokinase and ATP, it was shown, as expected (Zamecnik & Keller, 1954), that they were able to incorporate amino acids into protein for over 1 hr. (Fig. 5), but they were unable to incorporate amino acids into protein in the absence of ATP even when AMP and succinate were added (Fig. 5).

Effect of ribonuclease. Mitochondria were incubated in medium A with 0·5 mg. of ribonuclease/ml. at 25° for 30 min. in the presence of 10 mM-succinate. DL-[1-^{14}C]Leucine was then added and the mitochondria were incubated for a further hour at 37°. These mitochondria showed an incorporation of 240–270% of the incorporation shown by the control system of mitochondria that had been preincubated at 25° without ribonuclease.

Effect of antibiotics. Mitochondria were incubated in the normal system described, with the addition of 135 units of penicillin G/ml. and 18 μg. of chloramphenicol/ml. Incorporation of radioactive amino acids into the mitochondrial proteins was unimpaired.

Effect of preincubation of mitochondria with substrate. Mitochondria in medium B were preincubated at 37° for 30 min. in the presence of 10 mM-sodium succinate but in the absence of radioactive amino acid (Table 1). This treatment resulted in a reduction in the subsequent rate of incorporation of radioactively labelled amino acid into protein, compared with the rate of incorporation in the control mitochondria that had been stored at 0° during the time that the others were preincubated.

Content of RNA of the mitochondrial preparation. Since microsomal preparations give a high rate of amino acid incorporation *in vitro* it is important to show that microsomal contamination of the mitochondrial preparations is not significant.

The mean RNA content of 12 preparations of mitochondria as used in these experiments was 1·38 ± 0·12 (S.E.M.) mg. of RNA/100 mg. of protein. This amount could not be reduced by repeated resuspension of the mitochondria in 0·25 M-sucrose and resedimentation. Since the microsomal fraction is rich in RNA, the small amount of RNA in the mitochondria suggests that contamination by microsomes, if present, is slight.

When a preparation of mitochondria was incubated in medium A in the presence of 50 μg. of ribonuclease/ml. at 37°, the RNA content of the preparation fell to an average (four experiments) of 1·20 mg. of RNA/100 mg. of protein after incubation for about 30 min. and, thereafter, remains constant for incubation periods of up to 4 hr.

The values for RNA content of mitochondria obtained by various authors are compared in Table 4.

Effect of washing the mitochondria. When mitochondria were washed by resuspension and resedimentation in 0·25 M-sucrose the amino acid incorporation subsequently obtained during incubation under the conditions described was increased with each of the first four washes. The RNA content of the preparations fell simultaneously (Fig. 6).

Table 4. *Ribonucleic acid content of rat-liver mitochondria reported by various authors*

Reference	RNA (mg./100 mg. of protein)
Siekevitz & Watson (1956)	0·7
Laird, Nygaard, Ris & Barton (1953)	0·96
McLean, Cohn, Brandt & Simpson (1958)	1·0
Harel, Jacob & Moule (1957)	1·14
Muntwyler, Seifter & Harkness (1950)	1·36
Hogeboom, Schneider & Pallade (1948)	1·38
This paper	1·39
Roodyn, Reis & Work (1961)	1·4

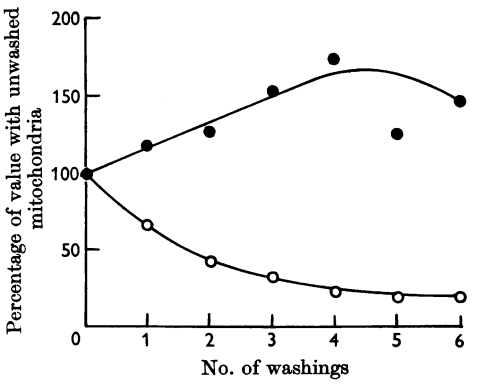

Fig. 6. Effect of washing on mitochondrial incorporation of amino acids into protein and on RNA content. ○, Total RNA of suspension; ●, incorporation into protein after incubation for 1 hr. in medium A plus 10 mM-ATP, 10 mM-sodium succinate, 0·2 ml. of cell sap/ml. of incubation medium and 0·5 μC of DL-[1-^{14}C]leucine/ml.

After eight washes, amino acid incorporation into mitochondrial protein was higher than in the unwashed preparation, even though the mitochondria were no doubt damaged to some extent by the resuspension and resedimentation.

The efficiency of the standard method of washing the mitochondria in removing microsomes was tested in another way by the following experiment. Microsomes obtained from a rat-liver homogenate were incubated for 20 min. with DL-[1-^{14}C]leucine in the presence of ATP, creatine, phosphate, creatine phosphokinase and cell sap. The labelled microsomes were isolated again and found to have a specific activity of 434 counts/min./mg. of protein; they were mixed with cell sap and mitochondria and the mitochondria were isolated from this simulated homogenate by the usual procedure and were washed. The radioactivity of the mitochondria was assessed at each stage of the preparative procedure and the microsomal contamination of the mitochondria calculated from the formula: $C = (1000R/S)/(P-R/S)$, where C is contamination (μg. of microsomal protein/mg. of mitochondrial protein), R is radioactivity of sample (counts/min.), S is specific activity of microsomal protein (counts/min./mg.), P is total protein of sample (mg.). The results are shown in Table 5. In the simulated homogenate the microsomes and mitochondria were present in roughly equal amounts. In the unwashed mitochondria contamination was reduced tenfold and after two washes the contamination by microsomes was estimated to be about 0·5%. The estimates of contamination after the four to six washes usually given were not reliable because by this stage the radioactivity was too slight to be determined accurately.

A third experiment to test for contamination by microsomes was carried out, by testing for glucose 6-phosphatase, which was shown by de Duve *et al.* (1955) to occur in high concentration in the microsomal fraction of rat liver. No glucose 6-phosphatase could be detected in a typical washed preparation of mitochondria by the method of Hers, Berthet, Berthet & de Duve (1951), although a high activity was found in a microsomal preparation from the same homogenate.

Damage to mitochondria during washing. It is probable that the rigorous washing procedure necessary to remove microsomes damages the mitochondria. Greville & Chappell (1959) report that in intact mitochondria only 8% of the total rhodanese activity of the mitochondria is free, the rest being latent and released when the mitochondria are disrupted. When mitochondria prepared in the way described here were examined, with the rhodanese assay as described by Greville & Chappell (1959), the free rhodanese detected in six preparations averaged 78% of the total rhodanese found when the mitochondria were disrupted by repeated freezing and thawing, indicating that the mitochondria are damaged.

The respiratory control ratios and P:O ratios of the mitochondrial preparations were also examined. With succinate as the substrate and ADP as the phosphate acceptor the respiratory control ratio averaged 4·91 and the P:O ratio 2·42, which are reasonable figures for a preparation of rat-liver mitochondria. It was concluded that although the large amount of free rhodanese suggests that the mitochondria are damaged, their respiratory functions are intact.

Free amino acids of the mitochondria. The average content of free amino acids of six preparations of mitochondria was 4·50 μg. of amino nitrogen/mg. of protein. The amino acids were analysed and found to occur in the proportions shown in Table 6.

Table 5. *Estimation of microsomal contamination*

Labelled microsomes with a specific activity of 434 counts/min./mg. of protein were mixed with cell sap and mitochondria from an homogenate. Mitochondria were then isolated and washed by resuspension and resedimentation in 0·25 M-sucrose. Samples were taken at each stage, and the amount of microsomes was estimated by measurement of radioactivity (see text).

Fraction	Microsomes (μg./mg. of mitochondria)
Total 'homogenate'	808
Unwashed mitochondria	80
Once-washed mitochondria	14
Twice-washed mitochondria	5
Three-times-washed mitochondria	5
Four- to eight-times-washed mitochondria	<5*

* Cannot be determined with accuracy; see text.

Table 6. *Free amino acids of mitochondria*

	Amino acid (μmoles/mg. of total amino N)
Alanine	7·59
Arginine	0·16
Aspartic acid	1·99
Cysteine	0
Glutamic acid	7·40
Glycine	11·95
Histidine	2·34
Isoleucine	0·47
Leucine	1·03
Lysine	3·55
Methionine	0·37
Phenylalanine	0·44
Proline	0
Serine	8·57
Threonine	1·55
Tyrosine	0·62
Valine	0·63

DISCUSSION

In view of the low rate of incorporation of amino acids into proteins obtained with the mitochondrial system in the work described in this paper and the work of others (McLean *et al.* 1958; Roodyn *et al.* 1961) and the much higher rates of incorporation that have been obtained with microsomal systems (Zamecnik & Keller, 1954) and with bacteria (Melchior, Mellody & Klotz, 1948), it is important to establish that the mitochondria are not significantly contaminated with either microsomes or bacteria.

The experiments reported here supply evidence that there is no serious contamination by microsomes. The low RNA content of the mitochondria, the lack of correlation between RNA content and ability to incorporate amino acids into protein (see Fig. 6) and the failure of ribonuclease to inhibit the incorporation (Table 1; McLean *et al.* 1958; Roodyn *et al.* 1961) suggest that microsomes are not responsible for the incorporation. The differences which occur in the optimum conditions for incorporation with microsomes and with mitochondria provide further evidence for the independence of the two systems. In the presence of ATP, Mg^{2+} ions were found to be inhibitory for the mitochondrial system (see Fig. 3) but they are necessary for the microsomal system (Sachs, 1957).

The time-curve of incorporation (Fig. 1) strongly suggests that bacterial contamination is not significant, since bacterial growth under the incubation conditions used would lead to a linear incorporation rate or to an increasing rate of incorporation. Similarly, preincubation at 37° would be expected to increase the rate of amino acid incorporation if bacteria were responsible for it, but such preincubation has been found to reduce incorporation (Table 1). The fact that incorporation occurs in the presence of antibiotics also suggests that bacteria are not responsible for the incorporation that was found.

Artifacts of labelling. The amount of amino acid incorporation into mitochondria is small but it probably represents a true biosynthesis of protein, for the incorporation is dependent on a supply of energy by oxidative phosphorylation and responds to changes which affect the energy metabolism of mitochondria, such as the uncoupling of oxidative phosphorylation with Ca^{2+} ions or the stimulation of oxidative phosphorylation by the addition of serum albumin. This dependence of the amino acid incorporation on the energy production of the mitochondria suggests that the process of incorporation is a biological one. The process used for preparing the protein samples is designed to remove adsorbed amino acids, RNA and lipids, and the average specific activity of the samples taken at the beginning of the incubations was only 0·25 counts/min./mg. of protein.

Causes of the low rate of incorporation. The incorporation obtained in the experiments described here is of the same order as that obtained by other workers with rat-liver mitochondria (McLean *et al.* 1958; Roodyn *et al.* 1961). Bates & Simpson (1959) reported high specific activities obtained after incubating calf-heart mitochondria with radioactive amino acid for periods of 12–16 hr., but Simpson, Skinner & Lucas (1961) now report that they can obtain incorporation only for periods of 3–4 hr. One possible cause of the low rate of incorporation into mitochondrial protein is that the size of the amino acid pool of the mitochondria leads to dilution of the isotopically labelled amino acids and hence to a reduction in the amount of radioactivity incorporated.

Fletcher & Sanadi (1961) report that rat-liver mitochondrial proteins have a half-life of about 10 days *in vivo*, and, if mitochondria are only engaged in the synthesis of their own proteins and not of proteins for transfer to other parts of the cell, then it is to be expected that mitochondrial proteins will become radioactively labelled at a very slow rate during turnover of the proteins.

It is widely accepted that proteins are synthesized on a template of RNA in ribonucleoprotein particles (Loftfield, 1957), and Rendi (1959) has described such particles from rat-liver mitochondria. The lack of inhibition of mitochondrial amino acid incorporation by ribonuclease and the failure of ribonuclease to hydrolyse more than a small portion of the RNA of the mitochondria may be explained by the protection of the ribonucleoprotein particles from the action of the ribonuclease by the mitochondrial membrane (Rendi, 1959). If the incorporation of radioactive amino acids into protein in mitochondria is expressed in terms of the RNA content, typical experiments give specific activities of 500–1000 counts/min./mg. of RNA, which is of the same order as that obtained with ribosomes (Korner, 1961). It may well be therefore that the RNA of mitochondria is as active in protein synthesis as is that of the microsomes, but that the low rate of amino acid incorporation in mitochondria is accounted for by their low RNA content.

SUMMARY

1. Isolated rat-liver mitochondria incorporate labelled amino acids into protein when incubated with an oxidizable substrate, a supply of adenine nucleotide and in the presence of PO_4^{3-}, Mg^{2+}, K^+ and Na^+ ions. Addition of cell sap was not necessary provided that a mixture of amino acids was supplied.

2. The concentrations of Mg^{2+}, K^+ and Na^+ ions

that were optimum for amino acid incorporation were determined.

3. Evidence is produced that the amino acid incorporation into protein of mitochondria was not caused by contaminating microsomes or bacteria.

4. The incorporation of amino acids into mitochondrial proteins was stimulated by preincubation of the mitochondria with ribonuclease.

5. The rate of incorporation of amino acids was shown to be correlated with the efficiency of the production of energy by oxidative phosphorylation.

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REFERENCES

Bates, H. M. & Simpson, M. V. (1959). *Biochim. biophys. Acta*, **32**, 597.
Chance, B. & Williams, G. R. (1955). *Nature, Lond.*, **175**, 1120.
Chappell, J. B. (1961). In *Biological Structure and Function*, vol. 2, p. 71. Ed. by Goodwin, T. W. & Lindberg, O. London: Academic Press Inc.
Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 111.
de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Dounce, A. L., Witter, R. F., Monty, K. F., Pate, S. & Cottone, M. A. (1955). *J. biophys. biochem. Cytol.* **1**, 139.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **43**, 190.
Fletcher, M. J. & Sanadi, D. R. (1961). *Biochim. biophys. Acta*, **51**, 356.
Greville, G. D. & Chappell, J. B. (1959). *Biochim. biophys. Acta*, **33**, 267.
Harel, L., Jacob, A. & Moule, Y. (1957). *Bull. Soc. Chim. biol.* **39**, 819.
Hers, H. G., Berthet, J., Berthet, L. & de Duve, C. (1951). *Bull. Soc. Chim. biol.* **33**, 21.
Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.
Korner, A. (1961). *Biochem. J.* **81**, 168.
Korner, A. & Debro, J. E. (1956). *Nature, Lond.*, **178**, 1067.
Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.
Laird, A. K., Nygaard, Q., Ris, H. & Barton, A. D. (1953). *Exp. Cell Res.* **5**, 147.
Laudahn, G. (1960). *Experientia*, **16**, 444.
Loftfield, R. B. (1957). *Progr. Biophys. biophys. Chem.* **8**, 347.
McLean, J. R., Cohn, G. L., Brandt, I. K. & Simpson, M. V. (1958). *J. biol. Chem.* **233**, 657.
Melchoir, J. B., Mellody, M. & Klotz, I. M. (1948). *J. biol. Chem.* **174**, 81.
Muntwyler, E., Seifter, S. & Harkness, D. M. (1950). *J. biol. Chem.* **184**, 181.
Rees, K. R. & Rowland, G. F. (1961). *Biochem. J.* **78**, 89.
Rendi, R. (1959). *Exp. Cell Res.* **17**, 585.
Roodyn, D. B., Reis, P. J. & Work, T. S. (1961). *Biochem. J.* **80**, 9.
Sachs, H. (1957). *J. biol. Chem.* **228**, 23.
Sacktor, B. (1953). *J. gen. Physiol.* **37**, 343.
Schneider, W. C. (1946). *J. biol. Chem.* **161**, 83.
Siekevitz, P. & Watson, M. L. (1956). *J. biophys. biochem. Cytol.* **2**, 653.
Simpson, M. V., Skinner, D. M. & Lucas, J. M. (1961). *J. biol. Chem.* **236**, PC 81.
Stern, H. & Timonen, S. (1955). *Exp. Cell Res.* **9**, 101.
Tsanev, R. & Markov, G. G. (1960). *Biochim. biophys. Acta*, **42**, 442.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.

Biochem. J. (1962) **83**, 596

The Effect of Salicylate on Adenosine-Triphosphatase Activity of Rat-Liver Mitochondria

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Although salicylic acid and its derivatives were first used in clinical medicine more than 80 years ago (Maclagen, 1876), very little is known of the mechanism by which they exert their wide variety of therapeutic and toxic effects. When salicylates are given in therapeutic dosage to the whole animal they produce a rapid but transient increase in metabolic rate (Austen, Rubini, Meroney & Wolff, 1958; Hetzel, Charnock & Lander, 1959). Salicylates can uncouple the oxidative phosphorylation of isolated mitochondria in concentrations that are within the range obtained in salicylate

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